Fixing Proteins on Electrophoresis Gels

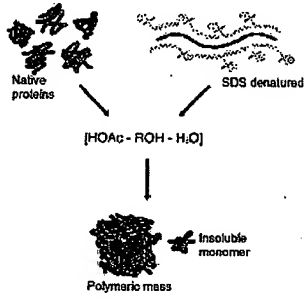

Figure 4.2.1a Fixing proteins with acetic acid and alcohol results in an uncoiling of the peptide chains to produce insoluble complexes and monomers.

Fixing (or fixation) is the process whereby proteins are denatured and precipitated in large insoluble aggregates within the gel matrix. Fixation accomplishes several goals. Primarily, fixation prevents the diffusion of proteins, thus keeping the protein bands sharp and resolved during the staining process. In addition, fixation removes gel buffer components, most importantly SDS, which may interfere in the staining process. In some cases, fixatives are used which modify the proteins to enhance the staining reaction.

An ideal fixative is fast, convenient and nonhazardous to use, and preserves the fine detail of the gel. It is important to be aware that fixing a protein within a gel drastically lowers the amount of protein which can be recovered from that gel after bands have been identified (see guide strip technique, Section 4.2.2). This is probably due to the trapping of gel matrix strands within the denatured protein complexes.

All fixatives operate by causing precipitation of the protein by converting it to an insoluble form. The most commonly used fixatives are solutions of short chain alcohols and acetic acid in water. The combination of low pH and high organic solvent content disrupts the hydrogen bonding which holds protein structures together, and exposes hydrophobic portions of the protein core. The result is an uncoiling of the peptide chain, followed by an essentially irreversible association between chains, producing a high molecular weight complex which is trapped inside the gel. This family of fixatives is cheap and relatively nonhazardous (depending on the alcohol used), and has the additional advantage that many stains are soluble in the fixative. This allows the combination of fixing and staining in one step. The only major drawback is that these solutions are only moderately denaturing, and may not fully fix small or unusually soluble proteins.

Stronger fixatives include trichloroacetic acid (12% in water), sulfosalicylic acid, or formaldehyde. TCA, sulfosalicylic acid and other strong acids act by protonating weak acids in the protein structure, disrupting the salt bridges and charge interactions required to maintain protein secondary structure. Aldehydes, such as formaldehyde and glutaraldehyde, react with amines on the surface of proteins, creating covalent cross links between protein molecules, resulting in a truly irreversible denaturation.

Appendix A

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Fixing Difficult Proteins

Small or unusually soluble proteins may not be sufficiently fixed by the above protocol. As these proteins diffuse through and out of the gel, smeared bands and loss of sensitivity may result. Prefixing of the gel in 12% trichloroacetic acid for 1-3 hours at room temperature prior to fixing by the above protocol will generally improve the fixing, and hence the staining of such proteins.

In certain cases, where proteins are heavily glycosylated or strongly basic, acid based fixatives may be ineffective. Small peptides may also be resistant even to strong acid fixatives. In such cases an effective alternative to acid precipitation is covalent cross-linking of the proteins with formaldehyde or glutaraldehyde. Formaldehyde fixation may be accomplished in a solution of 25% Ethanol, 15% Formalin (Formalin is 35% formaldehyde), 60% water. Gels are submerged in this solution for 1 hour, and may then be stained with or without subsequent alcohol/acetic acid fixation. Glutaraldehyde is generally used as a fixative in Silver Staining. Gels are soaked in 10% aqueous glutaraldehyde for 30 minutes, then washed for 2 x 20 minutes with water before staining. This denatures the proteins and fixes them in the gel; it also puts reactive aldehyde groups on the surface of the proteins, which enhance the silver stain reaction.

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Technical Library

Optimization of Southern Blotting Performance on Positively Charged Nylon Membranes

Protocol PC032

Michael A. Mansfield and Constance G. MacDonald

Abstract

Charged nylon membranes are commonly used as a support for hybridization of nucleic acids, but the parameters for optimal blotting performance have been poorly understood. In this poster, we describe optimization of DNA transfer and UV cross-linking conditions on Immobilon-Ny+. Remarkable enhancement of sensitivity and re-probing ability is thus realized. In terms of signal strength, alkaline transfer is shown to be inferior to standard transfer in 20X SSC, although either method can be utilized. UV cross-linking is the best method for fixing DNA to nylon membranes as this process results in covalent attachment of the DNA to the nylon. In this study, the performance of Immobilon-Ny+ in Southern blotting was analyzed as a function of transfer conditions and optimization of UV cross-linking. In addition sensitivity and reprobing characteristics were compared to other commercially available charged nylon membranes.

Materials and Methods

Electrophoresis and Capillary Blotting.

Lambda Hind III fragments were resolved electrophoretically on agarose and blotted to Immobilon-Ny+ (positively charged nylon membrane, 0.45 µm, Millipore) overnight by capillary transfer. The blots were dried prior to UV fixation.

UV Fixation of DNA. Transfer, cross-linking and stripping protocols are described in more detail in Millipore Technical Notes TN054, TN055 and TN056. Transferred DNA was UV cross-linked to the membrane at 254 nm using a Stratalinker (Stratagene Cloning Systems, La Jolla, California, USA) after drying the membranes.

Hybridization. Pre-hybridization was done for 2 h at 68°C; then hybridization solution containing 32P-labeled probe (Hind III DNA fragments) was added and incubated for 16 to 20 h at 68°C.

Imaging. Radioactivity on the membranes was visualized by phosphor imaging on a Storm 840 Phosphor Imaging System (Molecular Dynamics, Sunnyvale, California, USA); then quantified using ImageQuant analysis software.

Appendix B

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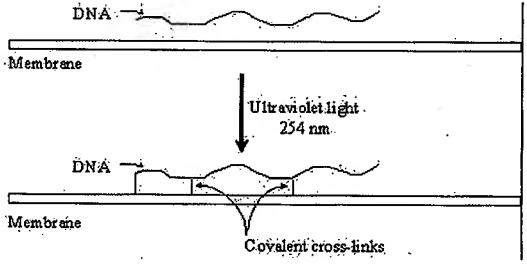
UV Cross-linking of DNA on Immobilon-Ny+



Comparison of SSC and Alkaline Transfer on Immobilon-Ny+

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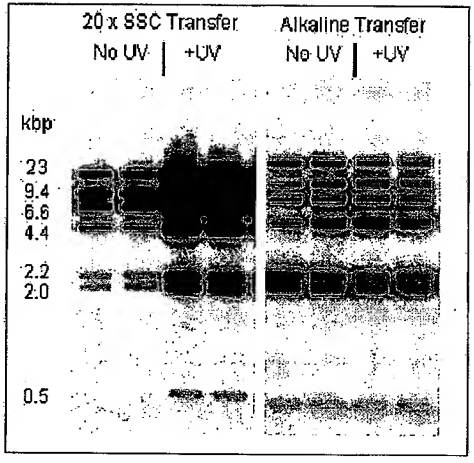

Relationship Between DNA Retention and Hybridization Signal Intensity

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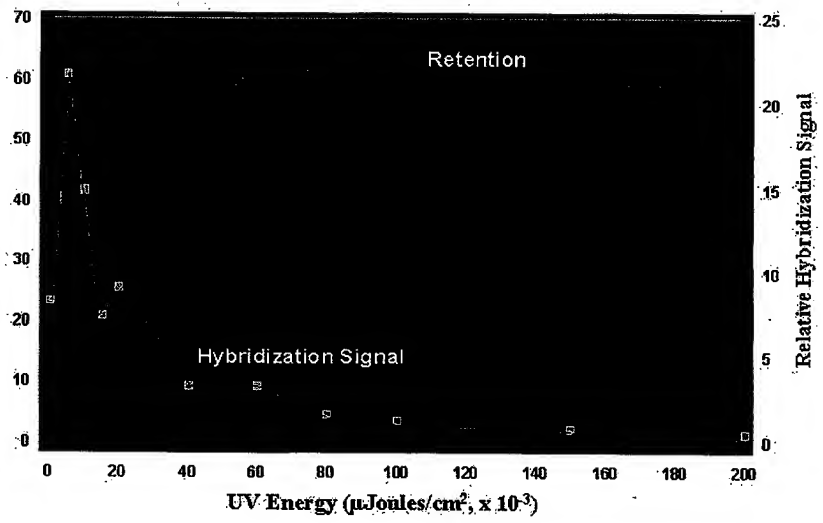

Effect of UV Energy on Hybridization Signal Intensity

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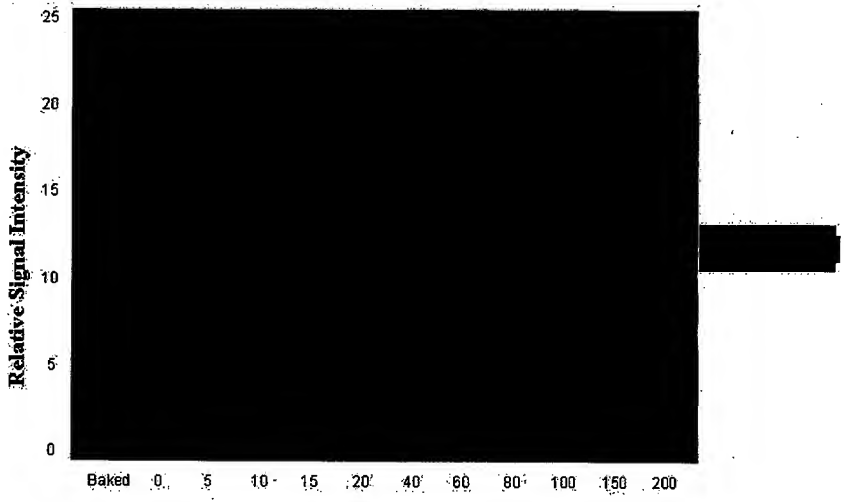

Change in Hybridization Signal with Multiple Reprobes

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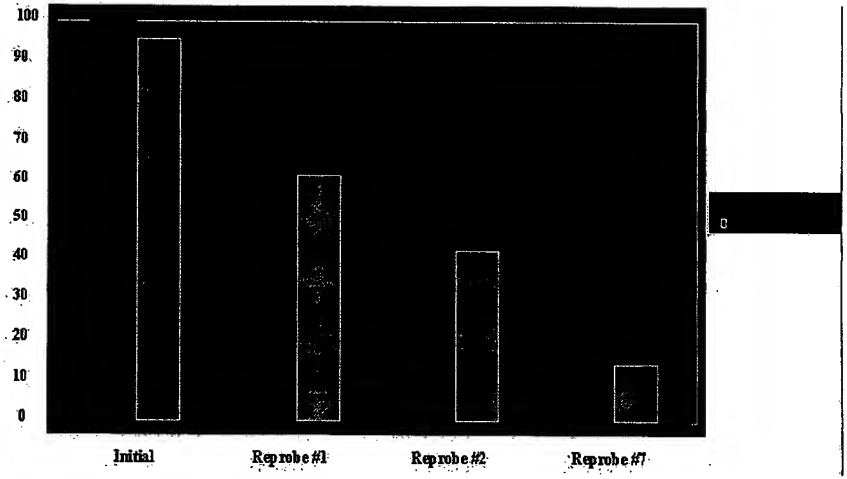

Reprobing of Immobilon-Ny+, Round 13

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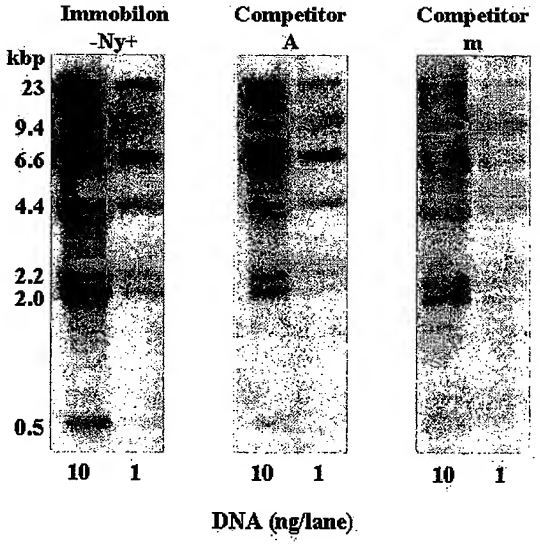

Conclusions

The efficiency of all nucleic acid hybridization assays on membranes is dependent on four major factors: elution of target DNA from the gel during transfer, binding of target DNA to the membrane during blotting, retention of target DNA during hybridization and stringency washes, and accessibility of the target DNA to the probe molecule. Without guidelines for fixation of target DNA to blotting membranes, DNA can be under-cross-linked and the target lost, or, over-cross-linked and the target rendered inaccessible to the probe. For optimal blotting performance with Immobilon-Ny+, the recommendations below should be followed:

The DNA must be applied to the membrane in single-stranded form (i.e., denatured).

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Transfer is best done using 20 x SSC. Alkaline transfer is inferior, but still an option.

UV cross-linking is the preferred method for DNA fixation. We recommend 5,000 µJoules/cm2 for optimal hybridization sensitivity as higher energy levels cause a rapid loss of signal.

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h e ch c che c c g e c b c f c f g

Appendix C

UVP

UVP, Inc. 2066 W. 11th Street, Upland, CA 91786
Tel: (800) 452-3197 or (909) 946-3197
Fax: (909) 946-3597
E-Mail: uvp@uvp.com

Application Bulletin

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Tel: +44(0)1223-420022 Fax: +44(0)1223-420561
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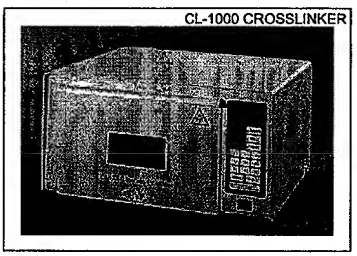
USES OF THE CL-1000 UV CROSSLINKER IN THE LABORATORY

The CL-1000 UV Crosslinker is a multi-purpose ultraviolet exposure instrument for use in the laboratory. Utilizing a 254nm shortwave ultraviolet (UV) radiation, the Crosslinker has the ability to perform a wide variety of applications.



INTRODUCTION

Ultraviolet radiation is a fast, easy, and effective method to fix nucleic acids to nitrocellulose, nylon, and nylon-reinforced membranes after Northern, Southern, slot or dot blotting. Ultraviolet radiation catalyzes the covalent attachment of nucleic acids to these membranes by activating interactions between thymines or uracils with the amine groups on the membrane matrix [1]. The result is higher resolution and sensitivity of subsequent hybridization analysis. The entire fixing process performed on the Crosslinker is around two to ten minutes, compared to the two hour period required for fixing by the baking method.

The Crosslinker is not limited to just fixing nucleic acid. The versatile wavelength is effective in many applications, compatible with most molecular biology experiments. CL-1000 UV Crosslinker can be used to nick ethidium bromide stained DNA in agarose gels, a step in alternating contour-clamped homogeneous electric field gel electrophoresis (CHEF) [2]. CHEF is a type of pulsed field gel electrophoresis, when used with the CL-1000 UV Crosslinker, can resolve DNA fragments greater than five megabases with clarity and ease.

UV irradiation of DNA provides an easy way to control the extent of restriction endonuclease digestion due to the fact that UV radiation dimerizes neighboring thymidines (TT, TTT, etc.). The restriction enzymes can not recognize and cleave the DNA if the thymidines within their restriction sites were dimerized [3]. The Crosslinker allows a greater control over partial digestions.

The Crosslinker offers a simple method to test for *recA* mutations. *RecA+* strains repair UV-induced damages and grow normally whereas mutations in *recA* prevent the cells to grow because of inability to repair damages [4]. By irradiating a strain with an unknown genotype, a mutation can be easily detect due to the properties mentioned above.

Ultraviolet radiation is often used in sterilization. The Crosslinker is effective in killing bacteria cultures, viruses, bacteriophages, and small organisms on surfaces [5]. It is an efficient alternative to the traditional heat germicide.

UV radiation can solve the problem of PCR (polymerase chain reaction) contamination. UV irradiated fragments form pyrimidine dimers which function as termination sites [6]. The formation of these termination sites eliminates most contamination caused by the reagents from the previous amplified material.

MATERIALS AND METHODS

A: FIXING NUCLEIC ACIDS ON TO MEMBRANES

Run agarose gel electrophoresis [7]. Transfer nucleic acids on to the membrane by capillary or other transfer methods [8]. Lay the membrane, nucleic acid side up, on a piece of tin foil. Do not cover. Turn the Crosslinker on. Press one of the

auto settings: UV energy exposure or time. Remove the membrane when the Crosslinker beeps five times. Continue with hybridization [9, 10].

B. NICKING ETHIDIUM BROMIDE STAINED DNA IN AGAROSE GELS

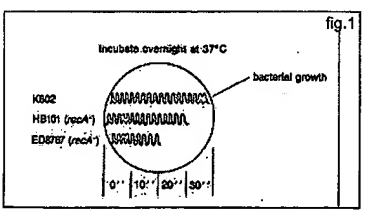
Prepare DNA samples. Run CHEF gel electrophoresis using a hexagonal array of electrodes. After electrophoresis, the gel is stained with ethidium bromide (0.5 µg/ml) and irradiated by the Crosslinker for about one minute [2]. The gel is then subjected to hybridization [10].

C. GENE MAPPING FOR CREATING CLEAVAGE-INHIBITING THYMINE DIMERS

Prepare DNA samples. Add 0.1-1 µg of DNA and 20 µl of restriction buffer. The restriction buffer must contain MgCl2. In the experiment conducted by Whittaker it was added into the mixture when the appropriate oligo was end-labeled. This mixture is irradiated for up to 60 minutes [3]. Then the mixture can be analyzed by agarose gel electrophoresis [7]. Longer irradiation time equals less cleavage by the restriction enzymes.

D. TESTING recA FUNCTION

Streak the strain being tested along with *recA+* strain on a petri dish. Us a piece of cardboard to cover about three quarters of each streak, expose the remaining portion to the Crosslinker for about ten seconds. Move the cardboard so half of the dish is covered. Expose the dish to the Crosslinker for ten seconds. Then move the cardboard so three forth of the petri dish is exposed, place it in the Crosslinker for ten seconds. Incubate the dish at 37°C overnight [4]. The *recA-* strain should be shriveled and shorter than the wild type strain (fig. 1).



E. ULTRAVIOLET STERILIZATION

Place the surfaces to be sterilized in to the Crosslinker for a set amount of time. Consult the Bacteria Destruction Chart on the UVP Internet home page for suggested time. Note: the Crosslinker cannot sterilize liquid nor solids.

F. ELIMINATION OF PCR CONTAMINATION

Irradiate the target DNA with the Crosslinker, five minutes is sufficient. For best results, the photoinduced defects should be in the sequence region bounded by the 3' ends of the PCR primers [11]. Add the PCR reaction components. Perform amplification as practiced [12].

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Strategy of Optimization for Silver Staining

Okt 27, 20

Droplet-Test | DNA-Silver | IEF-Silver | SDS-Silver

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General:

The pinciple of silver staining is the following:

In the gel the soluble, non visible Ag^+ -ions are reduced
to the metallic, black, and visible Ag^0.

The Ag+ donor is: $AgNO_3$

At the same time, the redox potential has to be chosen in that way, that the Ag+ ion, which is complexed in the polyacrylamide gel, can only react in the presence of an additional compound: a biomolecule like a protein or DNA.

The reducing solution: 0.037 % formaldehyde, and pH 12 due to 2.5 % sodium carbonate

To avoid re-desolving of precipitating Ag+-ions (brown, visible), such as AgCl, use:

Ag+ -complexing compound: 0.002 % sodium thiosulfate (only DNA silver-staining)

Appendix D

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Native-Elpho
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Solutions and Procedure (General, DNA-Silver-Staining)

A Fixing and Washing

At the beginning, the **gels** have to be fixed and washed. Different recipes for proteins and DNA.

Fixing DNA-gels: *15 % ethanol / 5 acetic acid with the gel swimming on the liquid, gel side looking downwar min at room temperature, 20 min when preheated to 50°C Washing with Bidest, gel swimming on the surface looking downward: 3 times 13 min at room temperature, 3 times 5 min when preheated to 50°C*
Newest recipe: Fixing with 0.6% Benzenesulfonic acid (free acid) [Merck 468] in 24% Ethanol

B The Silver Solution (prepare freshly)

0.1% $AgNO_3$ [Merck 1512] brings the Ag+ ions into the gel; stock solution: 2% > 10 ml silver solution + 200 formaldehyde (37%) [Merck 4003] per 200 mL Formaldehyd is the reducing reagent, but does not yet work, because the pH value is slightly acidic.

During the silvering, the gel lays at the bottom of the tray, gel side looks upward! 45 min at room temperatur min when preheated to 40°C

C The Developer (prepare freshly):

2.5 % Na_2CO_3 [Merck 6392] shifts the pH to 12, fast start, because formaldehyde is already present (silver solution!), stock solution: 10%. 0.037% formaldehyde reduces - together with the biomolecules - the Ag+ -io 0.002% sodium thiosulfate [Merck 6516] complexes precipitating Ag+ -ions (brown background!); recommen stock solution: 2%.>50 ml Na_2CO_3 + 200 µ l Formaldehyd + 150-200 µl Na-thiosulfate per 200 ml.

During the developing step, the gel lays at the bottom of the tray, gel side looks upward!3-6 min at room

h e ch c chee f ece h c h e h f g ge h :

temperature, dont heat! Best results when precooled to 8°C!

Testing and optimization of the 2 imortant solutions: The Droplet-Test (Button below)

D Stopping, Preserving and Drying

At the end, the **gels** have to be desilvered, preserved and dried.

.This can be done in one solution: 2% Glycin + 0.5 EDTA-Na. 20 min at room temperature. Air dry overnight

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